PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68, C07H 21/04

A1

(11) International Publication Number: WO 95/20055

(43) International Publication Date: 27 July 1995 (27.07.95)

(21) International Application Number: PCT/US95/00802

(22) International Filing Date: 19 January 1995 (19.01.95)

(30) Priority Data:
08/184,607 21 January 1994 (21.01.94) US

(71) Applicant: AMOCO CORPORATION [US/US]; Law Dept., 200 East Randolph Drive, P.O. Box 87703, Mail Code 1907A, Chicago, IL 60680-0703 (US).

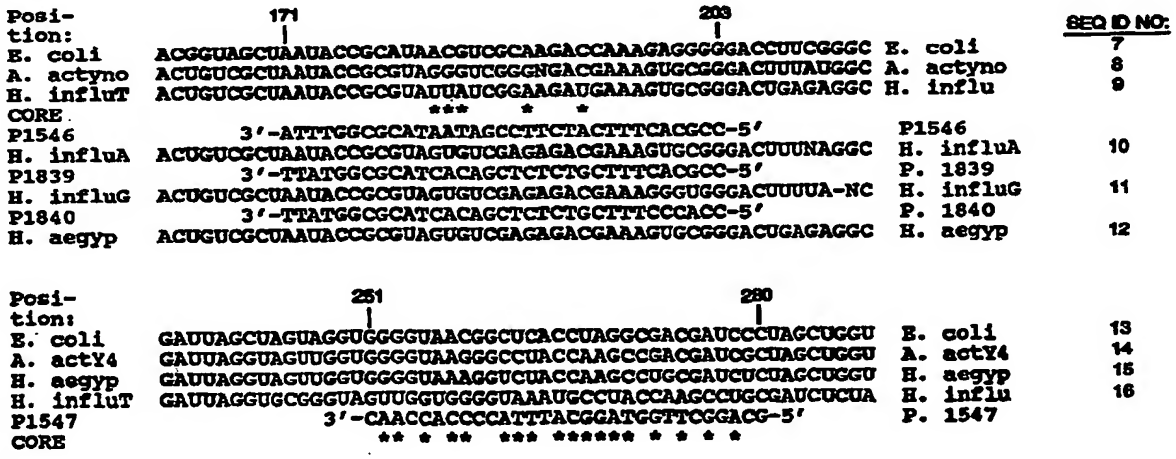
(72) Inventor: SHAH, Jyotsna; 14 Preserve Drive, Nashua, NH 03060 (US).

(74) Agent: TOLPIN, Thomas, W.; Amoco Corporation, Law Dept., Suite 600, 55 Shuman Boulevard, Naperville, IL 60563-8487 (US).

(81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: NUCLEIC ACID PROBES FOR THE DETECTION OF *HAEMOPHILUS INFLUENZAE*



(57) Abstract

Nucleic acids which hybridize to rRNA or rDNA of *Haemophilus influenzae* and methods for using these nucleic acids to detect the presence of *Haemophilus influenzae* in a sample are described. The figure is an illustration of the nucleotide sequences of regions 162 to 214 and 236 to 288 of the 16S rRNA of several *Haemophilus* species.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

NUCLEIC ACID PROBES FOR THE DETECTION OF *HAEMOPHILUS INFLUENZAE*

This invention relates to the detection of bacteria belonging to the species *Haemophilus influenzae*.

Background of the Invention

The genus *Haemophilus* includes bacteria classified as such, for example, in Bergey's *Manual of Systematic Bacteriology* (P.H.A. Sneath, ed., 1984, pp. 558-569, Williams & Wilkins). *Haemophilus* bacteria are minute to medium sized gram-negative coccobacilli or rods generally less than 1 μm in width and variable in length forming threads or filaments and showing marked polymorphism. They occur as obligate parasites on mucous membranes of man and a variety of animals. Capsules are present in a number of species and are of particular interest in *Haemophilus influenzae*, where they play a part in pathogenesis. The majority of *Haemophilus influenzae* strains can be assigned to any of six biovars (I-VI) on the basis of a few biochemical characteristics, and six serovars (A-F) have been identified on the basis of capsular polysaccharides. In addition, the species *Haemophilus aegyptius* has been shown to be the same species as *Haemophilus influenzae* (Casin et al., Ann. Microbiol. (Paris) 137B:155-163, 1986), and is now considered as another biovar of *H. influenzae* (Bergey's Manual of Determinative Biology, Hoft et al., eds., p. 195, Williams & Wilkins).

Haemophilus influenzae type b is a major cause of invasive diseases such as meningitis, epiglottiditis, arthritis, and cellulitis which occur exclusively in infants and children. In the United States, the annual incidence of invasive *Haemophilus influenzae* type b related diseases ranges from 67 to 129 cases per 100,000 children under 5 years of age, and the annual incidence of *Haemophilus*

meningitis is 19 to 69 cases per 100,000 children (Broom C.V., Pediatry Infect Dis 779-782, 1987). *Haemophilus influenzae* biovargroup aegyptius is mainly responsible for conjunctivitis, and certain strains are responsible for Brazilian purpuric fever (BPF), a serious invasive disease in children under 10 years of age (Brenner et al., J. Clin. Microbiol. 26:1524-1534, 1988).

The quick and accurate diagnosis of *H. influenzae* is important to treat the various diseases caused by this pathogen. However, most known diagnostic methods are slow or fairly non-specific. Diagnosis of *Haemophilus influenzae* is traditionally performed by microbacterial methods. Usually, Haemophilus colonies appear after overnight incubation on a selective chocolate 7% horse blood agar plate incubated at 37°C in moistened air supplemented with 7% carbon dioxide. Haemophilus colonies are then speciated and serotyped by biochemical tests for Gram strain morphology, oxidase reaction, hemolytic activity, porphyrin, and the requirement of growth factors X and V. Because of the time-consuming nature of these techniques, a number of rapid serological methods have recently become available. The vast majority are antibody-based tests, but most lack sufficient specificity for conclusive identification of the presence of *Haemophilus influenzae*. Therefore, it is the aim of the present invention to provide nucleic acid probes which allow the specific identification of *Haemophilus influenzae* in a sample in the presence of other bacteria or fungi which are normally present in sampled materials. Such probes may be used in a variety of inexpensive, easy-to-use assay systems which allow an accurate, rapid diagnosis of *Haemophilus influenzae* related diseases, and which avoid many of the disadvantages associated with traditional microbiological techniques.

Summary of the Invention

In one aspect, the invention features an isolated nucleic acid containing about 10 to 70 nucleotides which forms a stable hybridization duplex under normal hybridization conditions with a region of *Haemophilus influenzae* 16S rRNA or rDNA bounded by nucleotide positions 182 to 193 or 252 to 278 or, alternatively, to a region of *Haemophilus influenzae* 23S rRNA or rDNA bounded by nucleotide positions 343 to 356 (*E. coli* numbering system). Preferably the nucleic acid is between 20 and 60 nucleotides, and most preferably between 25 and 55 nucleotides in length, and is capable of hybridizing preferentially to rRNA or rDNA of *Haemophilus influenzae* over rRNA or rDNA of non-*Haemophilus* organisms. Most preferably, the nucleic acid is capable of hybridizing preferentially to rRNA or rDNA of *H. influenzae* over rRNA or rDNA of non-*H. influenzae* organisms.

In preferred embodiments of this aspect of the invention, the nucleic acid forms a stable hybridization duplex under normal hybridization conditions with probe 1546 (SEQ ID NO: 1), probe 1984 (SEQ ID NO: 5), or a nucleotide sequence which is complementary to either of these probes. Preferably, the nucleic acid contains at least 10, and more preferably at least 20 consecutive nucleotides which are homologous or complementary to probe 1546 (SEQ ID NO: 1) or probe 1984 (SEQ ID NO: 5). Most preferably, the nucleic acid is 1546 (SEQ ID NO: 1) or 1984 (SEQ ID NO: 5), or a nucleic acid complementary to one of these probes. In addition, individuals skilled in the art will readily recognize that nucleic acids are subject to modification and alteration, and accordingly, the present invention also includes nucleic acids which are complementary or homologous to at least 90% of any ten, and preferably any 20,

consecutive nucleotides within probe 1546 (SEQ ID NO: 1) or 1984 (SEQ ID NO: 5), and which have the hybridization specificity of these particular probes.

In another aspect, the invention features a set of at least two isolated nucleic acids wherein each nucleic acid is between 10 and 70 nucleotides, and forms a stable hybridization duplex under normal hybridization conditions with a region of *Haemophilus influenzae* 16S rRNA or rDNA bounded by nucleotide positions 182 to 193 or 252 to 278, or a region of *Haemophilus influenzae* 23S rRNA or rDNA bounded by nucleotide positions 305 or 314 or 343 to 356. Preferably each nucleic acid in the set is between 20 and 60 nucleotides, and most preferably between 25 and 55 nucleotides in length. In the event that one of the nucleic acids within the set also forms a stable hybridization duplex with rRNA or rDNA of a non-*Haemophilus influenzae* organism under normal hybridization conditions, a second nucleic acid within the set does not form a stable hybridization duplex with the rRNA or rDNA of the same non-*Haemophilus influenzae* organism under the same hybridization conditions.

In preferred embodiments of this aspect of the invention, the set of nucleic acids includes at least one nucleic acid which is capable of preferentially hybridizing to rRNA or rDNA of *Haemophilus influenzae* over rRNA or rDNA of non-*Haemophilus influenzae* organism. More preferably, at least one of the nucleic acids of the set consists essentially of the nucleotide sequence defined by probe 1546 (SEQ ID NO: 1), 1547 (SEQ ID NO: 2), 1839 (SEQ ID NO: 3), 1840 (SEQ ID NO: 4), 1984 (SEQ ID NO: 5), 23HI (SEQ ID NO: 6), or a nucleotide sequence complementary to any one of these probes. Especially preferred sets include probes 1546 (SEQ ID NO: 1) and 1547 (SEQ ID NO: 2); and 1984 (SEQ ID NO:

5) and 23HI (SEQ ID NO: 6). In addition, individuals skilled in the art will readily recognize that the sequences of probes 1546 (SEQ ID NO: 1), 1547 (SEQ ID NO.: 2), 1839 (SEQ ID NO: 3), 1840 (SEQ ID NO: 4), 1984 (SEQ ID NO: 5), 23HI (SEQ ID NO: 6), and their complementary sequences are subject to modifications and alterations. Accordingly, the nucleic acid set of the invention can include one or more nucleic acids which are complementary or homologous to at least 90% of any ten, and preferably any 20, consecutive nucleotides within probe 1546 (SEQ ID NO: 1), 1547 (SEQ ID NO: 2), 1839 (SEQ ID NO: 3), 1840 (SEQ ID NO: 4), 1984 (SEQ ID NO: 5) or 23HI (SEQ ID NO: 6) and which have the hybridization specificity of these particular probes.

The invention also features a method of detecting the presence of *Haemophilus influenzae* in a sample. The method involves contacting the sample with at least one of the nucleic acids of the invention under normal hybridization conditions which allow the formation of nucleic acid duplexes between the nucleic acid and rRNA or rDNA of *Haemophilus influenzae*, if present, and then monitoring the contacted sample to detect the presence of the nucleic acid duplexes wherein the presence of the duplexes is an indication that *Haemophilus influenzae* is present in the sample. The detection of the duplexes may involve any standard techniques for identifying duplex molecules. Preferably, either the nucleic acid of the invention, or the *Haemophilus influenzae* rRNA or rDNA are labeled with a chemical moiety which is capable of being detected, including without limitation, radioactive isotopes, enzymes, luminescent agents, precipitating agents and dyes.

In one preferred embodiment, the method involves contacting the sample with a set of at least two nucleic

acids each of which is capable of hybridizing to the rRNA or rDNA of *Haemophilus influenzae*. Preferred sets of probes include probes 1546 (SEQ ID NO: 1) and 1547 (SEQ ID NO: 2); and probes 1984 (SEQ ID NO: 5) and 23HI (SEQ ID NO: 6).

Individuals skilled in the art will readily recognize that the compositions of the present invention can be assembled in a kit for the detection of *Haemophilus influenzae*. Typically, such kits include reagents containing the nucleic acids of the present invention with instructions and suitable packaging for their use as part of an assay for *Haemophilus influenzae*.

The term "isolated nucleic acid," as used herein, refers to a nucleic acid segment or fragment which is not immediately contiguous with (i.e., covalently linked to) both of the nucleic acids with which it is immediately contiguous in the naturally occurring genome of the organism from which the nucleic acid is derived. The term, therefore, includes, for example, a nucleic acid which is incorporated into a vector (e.g., an autonomously replicating virus or plasmid), or a nucleic acid which exists as a separate molecule independent of other nucleic acids such as a nucleic acid fragment produced by chemical means or restriction endonuclease treatment.

A nucleic acid "consisting essentially of" a particular sequence of nucleotides as used herein refers to that particular sequence and other sequences that are identical to the first sequence but for the addition to or removal from the sequence of a few nucleotides (e.g. 2 to 10) which does not prevent the nucleic acid sequence from forming stable hybridization duplex with a particular region of *H. influenzae* rRNA.

"Homologous," as used herein in reference to nucleic acids, refers to the nucleotide sequence similarity between

two nucleic acids. When a nucleotide sequence is identical to another sequence, then the two sequences are 100% homologous. The homology between two nucleic acids is a direct function of the number of matching nucleotides at a given position in the sequence, e.g., if half of the positions in the two nucleic acids are the same then they are 50% homologous. Thus, "substantially homologous" means a nucleotide sequence that is not identical to another sequence, but maintains a sufficient sequence identity to retain the same hybridization characteristics as the sequence.

The term "complementary" means that two nucleic acids, e.g. DNA or RNA, contain a series of consecutive nucleotides which are capable of forming base pairs to produce a region of double-strandedness referred to as a hybridization duplex or complex. A duplex forms between nucleic acids because of the orientation of the nucleotides on the RNA or DNA strands; certain bases attract and bond to each other to form a base pair through hydrogen bonding and π-stacking interactions. Thus, adenine in one strand of DNA or RNA pairs with thymine in an opposing complementary DNA strand, or with uracil in an opposing complementary RNA strand. Guanine in one strand of DNA or RNA pairs with cytosine in an opposing complementary strand.

The formation of such hybridization duplexes can be made to be highly specific by adjustment of the conditions (often referred to as stringency) under which this hybridization takes place such that hybridization between two nucleic acids will not form a stable duplex, e.g., a duplex that retains a region of double-strandedness under the predetermined stringency conditions, unless they contain a certain number of nucleotides in specific sequences which are substantially, or completely, complementary. Thus, the

phrase "preferentially hybridize" as used herein refers to a nucleic acid which binds to, and forms a stable duplex under normal hybridization conditions with, a second nucleic acid (i.e., *Haemophilus influenzae* rDNA or rRNA), and which does not form a stable duplex with other nucleic acid molecules under the same normal hybridization conditions such as those described herein.

Unless defined otherwise, all technical terms and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications mentioned herein are incorporated by reference. Examples of the preferred methods and materials will now be described. These examples are illustrative only and not intended to be limiting as those skilled in the art will understand that methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Drawings

The drawings will first be briefly described.

Figure 1 is an illustration of the nucleotide sequences of regions 162 to 214 and 236 to 288 of the 16S RNA from several of the species examined. H. influT = *H. influenzae* type strain; H. influA = *H. influenzae* type A; H. influG = *H. influenzae* type G; *H. aegyT* = *H. aegyptius* type strain; A. actY4 = *A. actinomycemcomitans*. Probes 1546, 1839, 1840, and 1547 are also illustrated. RNA sequences are written 5′ to 3′. Probe sequences are written 3′ to 5′. The core region is bounded by *.

Figure 2 is an illustration of the nucleotide sequences of regions 280 to 335 and 335 to 372 of the 23S RNA from several of the species examined. H. influF = *H. influenzae* type strain E; H.; H. aegyT = *H. aegyptius* type strain. H. pleur = *H. pleuropneumo*; *H. parain* = *H. parainfluenza* and A. actyno = *A. actinomycetemcomitans*. Probes 23HI and 1984 are also illustrated. RNA sequences are written 5′ to 3′. Probe sequences are written 3′ to 5′. The core region is bounded by *.

Detailed Description

Bacterial ribosomes contain three distinct RNA molecules which, at least in *Escherichia coli*, are referred to as 5S, 16S and 23S rRNAs. In eukaryotic organisms, there are four distinct rRNA species, generally referred to as 5S, 18S, 28S, and 5.8S. These names historically are related to the size of the RNA molecules, as determined by their sedimentation rate. In actuality, however, ribosomal RNA molecules vary substantially in size between organisms. Nonetheless, 5S, 16S, and 23S rRNA are commonly used as generic names for the homologous RNA molecules in any bacterium, including *Haemophilus*, and this convention will be continued herein. The probes of the present invention target specific regions of the 16S and 23S rRNA molecules of the *Haemophilus influenzae*, and are useful to detect this pathogen in biological samples.

I. Identification of Unique Regions of the *Haemophilus influenzae* rRNA genes

The nucleic acid probes of the present invention were developed by comparing regions of *Haemophilus influenzae* rRNA and rDNA to the rRNA and rDNA of close evolutionary relatives based on phylogenetic observations.

As the first step in identifying regions of *Haemophilus influenzae* rRNA which could potentially serve as

useful target sites for nucleic acid hybridization probes, complete nucleotide sequences of the 16S rRNAs of *Haemophilus influenzae*, several other species of *Haemophilus*, and close relatives of *Haemophilus* were obtained, and partial sequencing of the 23S rRNAs through the "300 Region" (Gutell and Fox, 1988, *Nucleic Acid Research*, 16 r175-r269) from several serovars of *Haemophilus influenzae*, other species of *Haemophilus*, and *Actinobacillus actinomycetemcomitans* was performed.

The nucleotide sequences were determined by standard laboratory protocols either by cloning (Maniatis et al., 1982, *Molecular Cloning; A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, pp 545) and sequencing (Maxam and Gilbert, 1977, *P.N.A.S., USA* 74:560-564; Sanger et al., 1977, *P.N.A.S., USA* 74:5463-5467) the genes which specify the rRNAs, and/or by direct sequencing of the rRNAs themselves using reverse transcriptase (Lane et al., 1985, *P.N.A.S., USA* 82:6955-6959).

The *Haemophilus influenzae* rRNA nucleotide sequences were then compared to other available rRNA nucleotide sequences, in particular to other species of Haemophilus, and *Actinobacillus actinomycetemcomitans*. The *Escherichia coli* 16S and 23S rRNA sequences are used herein as a convenient standard for identifying particular homologous regions in the *Haemophilus* rRNAs under consideration.

Comparison of the sequences of different serotypes of *Haemophilus influenzae* and other *Haemophilus* revealed several regions of sequence which are different in the different serotypes of *Haemophilus influenzae*, and between *Haemophilus influenzae* and non-*Haemophilus influenzae*, and between *Haemophilus influenzae* and non-*Haemophilus* bacteria (Figures 1 and 2).

II. Development of Probes

A minimum of ten nucleotides are necessary in a nucleic acid probe to statistically obtain specificity and for stable hybridization products. Nucleic acids of greater length, up to 2500 nucleotides, have been suggested as probes. However, a maximum of 250 nucleotides presently represents an approximate upper limit for nucleic acid probes in which reaction parameters can be readily adjusted and controlled to determine mismatched sequences and preferential hybridization. The maximum of 250 nucleotides also represents the upper limit of most currently used synthetic methods of generating DNA and RNA molecules. Preferably, useful probes have between 20 and 70 nucleotides. A number of probes were designed to the target regions corresponding variously to complements of the sequences of *Haemophilus influenzae* serovars A, B, and G, or all three.

Oligonucleotide probes, 28-42 nucleotides in length, were designed which hybridize preferentially to *Haemophilus influenzae*. These were designed:

1) to maximally utilize the nucleotide sequence differences useful for distinguishing *Haemophilus influenzae* or *Haemophilus* from other bacteria,

2) to minimize the effect of self complementarity, both locally within the target rRNA, and between probes, and

3) to contain a "core region" of approximately 10 to 25 nucleotides to maximize specific hybridization to *Haemophilus influenzae* rRNA or rDNA over other species under normal hybridization conditions.

Specifically, the nucleotide sequence of the core region of each probe was designed to have the maximum number of nucleotide mismatches with the rRNA of other species while maintaining sufficient complementarity to allow

hybridization of the probe to Haemophilus influenzae rRNA. "Nucleotide mismatch" means that a nucleotide position in the probe is occupied by a nucleotide which does not normally form a base pair with the nucleotide at the corresponding position in the complementary target RNA or DNA. The preferred types of mismatches include uracil to uracil, adenine to adenine, guanine to guanine, cytosine to cytosine and thymine to thymine, but adenine to cytosine, uracil to cytosine and thymine to cytosine may also be used.

Single stranded probes were prepared using beta-cyanoethyl phosphoramidite chemistry on a 380-B Synthesizer (Applied Biosystems, Foster City, CA). Deprotection of the phosphates and nucleotide bases was accomplished by standard methods and the crude oligonucleotides mixtures were purified by reverse phase HPLC.

This probe selection strategy yielded a number of probes useful for identifying *Haemophilus influenzae* bacteria in samples. Probes 1546 (SEQ ID NO: 1), 1547 (SEQ ID NO: 2), 1839 (SEQ ID NO: 3) and 1840 (SEQ ID NO: 4) are designed from 16S rRNA sequences, and probes 1984 (SEQ ID NO: 5) and 23HI (SEQ ID NO: 6) are designed from 23S rRNA sequences. The probe sequences are set forth below.

16S rRNA-Targeted Probes:

Probe 1546: 5'-CCGCACTTTCATCTTCCGATAATACGCGGTATT-3' (SEQ ID NO: 1)
Probe 1547: 5'-GCAGGCTTGGTAGGCATTTACCCCACCAAC-3' (SEQ ID NO: 2)
Probe 1839: 5'-CCGCACTTTCGTCTCTCGACACTACGCGGTATT-3' (SEQ ID NO: 3)
Probe 1840: 5'-CCACCCTTTCGTCTCTCGACACTACGCGGTATT-3' (SEQ ID NO: 4)

23s rRNA-Targeted Probes:

Probe 1984: 5'-TTAGTACCACAATATGGTTTTTAGATAC-3' (SEQ ID NO: 5)
Probe 23HI: 5'-CAATCCTCTTACACAACCCTTCGTGTTAGTTTCTCCCACTAT-3' (SEQ ID NO: 6)

III. Hybridization Conditions

In general, hybridization conditions are defined by the base composition of the nucleic acid duplex, as well as

by the level and geometry of mispairing between the two nucleic acids. Reaction parameters which are commonly adjusted include concentration and type of ionic species present in the hybridization solution, the types and concentrations of denaturing agents present, and the temperature of hybridization. Generally, as hybridization conditions become more stringent, longer probes are preferred if stable hybrids are to be formed. As a corollary, the stringency of the conditions under which hybridization is to take place (e.g., based on the type of assay to be performed) will dictate certain characteristics of the preferred probes to be employed. Such relationships are well understood and can be readily manipulated by those skilled in the art (see for example, Ausebel et al. *Current Protocols in Molecular Biology,* John Wiley & sons, New York, 1989). For example, the appropriate hybridization conditions for any particular probe may be determined using the melting temperature (T_m) of the desired duplex molecule as a guide. The T_m of any DNA-DNA duplex may be approximated from the equation of Mein Koth and Wahl:

$$T_m = 81.5°C+16.6(\log M)+0.41(\%GC)-0.61(\%form)-500/2$$

and for RNA-DNA hybrids from the equation of Casey and Davidson:

$$T_m = 79.8°C+18.5(\log M)+0.58(\%GC)-11.8(\%GC)^2-0.56(\%form)-820/2$$

where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, %form is the percentage of formamide in the hybridization solution, an L is the length of the hybrid duplex in base pairs.

Generally, for the specific probes disclosed, normal hybridization conditions involve hybridization in a high salt solution that promotes base-pairing between the probe and target sequence at a temperature between 42°C and 68°C.

As is well known, the specificity of the particular probe is defined by the post-hybridization washes, which in the present case are typically carried out at 60°C-68°C at an ionic concentration which is ten to thirty times lower than the concentration used in the hybridization step.

The specific hybridization behaviors of the nucleic acid probes described above are dependent to a significant extent on the assay format in which they are employed. Conversely, the assay format will dictate certain optimal design features of particular nucleic acids. For example, the length of the particular oligonucleotides described herein was optimized for use in the dot blot assay but may be used in other standard hybridization assays. It is well known to one skilled in the art that optimal probe length will be a function of the stringency of the hybridization conditions chosen; hence the length of the instant probes may be altered in accordance with these conditions as long as the probe includes a sequence substantially homologous or complementary to the "core regions" defined herein. Also, in considering sets comprised of more than one nucleic acid probe, it is desirable that all probes behave in a compatible manner in any particular format in which they are both employed. Thus, the exact length of a particular nucleic acid probe will, to a certain extent, reflect its specific intended use.

IV. Hybridization Analysis of Probe Behavior

The sequence comparison suggested that the nucleic acid probes of the present invention should exhibit a variety of useful hybridization properties with respect to the specific detection of *Haemophilus influenzae* or other *Haemophilus* species, or both, to the exclusion of other bacteria. Equally as important as the inclusivity behavior

of the probes is their exclusivity behavior, i.e., their reactivity toward non-*Haemophilus* bacteria.

The behavior of the probes toward representative *Haemophilus influenzae* and non-*Haemophilus* bacteria was determined by hybridization analysis using a dot blot procedure. While hybridization data for each of the individual probes of the present invention are provided (Tables 1 and 2), it should be noted that useful combinations (sets) of probes which exhibit hybridization behaviors that are the sum of the individual probes also is explicitly predicted by the data.

Example 1: Dot blot analysis of probe hybridization behavior

Dot blot analysis, in accordance with well known procedures, involves immobilizing a target rRNA or rDNA or a population of target nucleic acids on a filter such as a nitrocellulose, nylon, or other derivatized membrane which readily can be obtained commercially, specifically for this purpose. Either DNA or RNA can be easily immobilized on such a filter and subsequently tested for hybridization under any of a variety of conditions (i.e., stringencies) with the nucleic acid probes of the invention. Under controlled conditions, probes whose nucleotide sequences have greater complementarity to the target sequence will exhibit a higher level of hybridization than probes having less complementarity.

One tenth of a microgram of purified RNA (Lane et al., 1985, *P.N.A.S., USA* 82:6955-6959), or cells from each of the indicated organisms, was spotted on nitrocellulose filters. The oligonucleotide probes were end-labeled with radioactive phosphorous 32 using standard procedures.

Hybridization to the rRNA targets at 60°C for 14-16 hours (in a hybridization solution containing 0.9 M NaCl, 0.12 M Tris-HCl, pH 7.8, 6mM EDTA, 0.1 M KPO_4, 0.1% SDS, 0.1% pyrophosphate, 0.002% ficoll, 0.02% BSA, and 0.002% polyvinylpyrrolidine), followed by three 15 minute post-hybridization washes at 60°C (in 0.03 M NaCl, 0.004 M Tris-HCl, pH 7.8, 0.2 mM EDTA, and 0.1% SDS) to remove unbound probes, was found to be sufficiently stringent to produce a high level of probe specificity.

Following hybridization and washing as described above, the hybridization filters were exposed to x-ray film overnight with two intensifying screens, and the intensity of the signal was scored visually with respect to control spots of known amount of target material (RNA). A signal of four positive signs (++++) indicates a high level of hybridization, and this level was used as a standard. In comparison to this standard or control level, two positive signs (++) indicates a faint level of hybridization, one positive sign (+) a level that is barely detectable, and a negative (-) sign indicates that hybridization was undetectable.

Table 1 exemplifies the inclusivity behavior of the preferred probes toward a representative sampling of the different serotypes of *Haemophilus influenzae* as well as from the biovar *aegyptius* in a dot blot hybridization assay. Either purified RNA or cells were tested. As used in Table 1, "Cyto-dots" are cells lysed onto nitrocellulose by standard NaOH treatment, followed by neutralization with Tris buffer pH 7.4.

TABLE 1: DOT-BLOT HYBRIDIZATION OF 16S AND 23S rRNA-TARGETED PROBES (INCLUSIVITY TESTING)

Genus, species	Target: / Probe: strain	16S 1546	16S 1547	16S 1839	16S 1840	23S 1984
RNA						
Haemophilus influenza	GT0244	++++	++++	+++	++++	++++
Haemophilus influenza A	GT2563	-	++++	++++	+++	++++
Haemophilus influenza B	GT2564	++++	++++	-	+	++++
Haemophilus influenza B	GT2565	++++	++++	-	+	++++
Haemophilus influenza C	GT2566	++++	++++	++++	+++	++++
Haemophilus influenza D	GT2567	++++	++++	-	+	++++
Haemophilus influenza E	GT2568	++++	++++	+++	+++	++++
Haemophilus influenza F	GT2569	-	++++	++++	++++	++++
Haemophilus influenza	ATCC33391	++++	++++	++++	++++	++++
Haemophilus aegyptius	NCTC8052	-	++++	++++	++++	++++
Cyto-dots						
Haemophilus influenza	GT0244	++++	++++	++	++++	++++
Haemophilus influenza A	GT2563	-	++++	+	++	++++
Haemophilus influenza B	GT2564	++++	++++	-	+	++++
Haemophilus influenza B	GT2565	++++	++++	-	+	++++
Haemophilus influenza C	GT2566	++++	++++	++++	++++	++++
Haemophilus influenza D	GT2567	++++	++++	-	+	++++
Haemophilus influenza E	GT2568	++++	++++	+	++	++++
Haemophilus influenza F	GT2569	-	++++	+	++++	++++
Haemophilus influenza	GT1367	-	++++	+	++	++++
Haemophilus influenza	GT1372	-	++++	+	++++	++++
Haemophilus influenza	GT1680	++++	++++	-	+	++++
Haemophilus influenza	GT1861	++++	++++	+	++++	++++
Haemophilus influenza	GT1862	++++	++++	-	+	++++
Haemophilus influenza	GT1882	++++	++++	-	+	++++
Haemophilus influenza	GT1883	++++	++++	-	+	++++
Haemophilus influenza	GT1884	++++	++++	-	+	++++
Haemophilus influenza	GT1885	++++	++++	-	+	++++
Haemophilus influenza	GT1886	++++	++++	-	+	++++
Haemophilus influenza	GT1948	++++	++++	-	+	++++
Haemophilus influenza	GT1949	++++	++++	-	+	++++
Haemophilus influenza	GT1950	++++	++++	-	+	++++
Haemophilus influenza	GT1951	++++	++++	+++	++++	++++
Haemophilus influenza	GT1973	++++	++++	-	+	++++
Haemophilus influenza	GT2016	++++	++++	-	+	++++
Haemophilus influenza	GT2017	-	++++	++++	++++	++++
Haemophilus influenza	GT2058	++++	++++	-	+	++++
Haemophilus influenza	GT2059	++++	++++	-	+	++++
Haemophilus influenza	GT2130	nd	++++	-	+	++++
Haemophilus influenza	GT2156	-	++++	-	-	++++

100 ng RNA or 5ul of cells (10E10) were spotted per dot.
Results were recorded after overnight exposure of autoradiographs with 2 intensifying screens. nd = not done
++++ = control level of hybridization; + = barely visible; and - = zero

Table 2 exemplifies the exclusivity behavior of the preferred probes toward a representative sampling of non-*Haemophilus* bacteria in a dot blot hybridization assay.

TABLE 2: DOT-BLOT HYBRIDIZATION OF 16S AND 23S rRNA-TARGETED PROBES (EXCLUSIVITY TESTING)

	Target:	16S				23S
Genus, species	Probe: strain	1546	1547	1839	1840	1984
RNA						
Haemophilus influenza	ATCC33391	++++	++++	++++	++++	++++
Haemophilus aphrophilus	ATCC33389	+/-	++++	+	++	-
Haemophilus aviam	NCTC11297	-	++++	-	+	-
Haemophilus ducreyii	GT0243	-	-	-	-	-
Haemophilus haemoglobin	NCTC1659	-	-	-	+	-
Haemophilus parainfluenza	ATCC29241	-	-	-	+	-
Haemophilus parainfluenza	GT1368	-	-	-	-	-
Haemophilus parainfluenza	GT2131	-	++++	-	+	-
Haemophilus parainfluenza	GT2193	-	-	-	-	-
Haemophilus pleuropneumo.	ATCC27088	-	-	-	+	-
Haemophilus segnis	ATCC33393	-	+++	++++	+++	-
A. actinomycetemcomitans	GT0004	-	+++	++++	++	-
A. actinomycetemcomitans	ATCC29522	-	+++	++++	++	-
A. actinomycetemcomitans	ATCC29524	-	++++	++++	++	-
A. actinomycetemcomitans	ATCC29523	-	++++	++++	++	-
A. seminis	ATCC15768	-	++++	-	++++	-
A. equuli	NCTC08829	-	+++	-	+	-
A. ligieresii	ATCC19393	-	-	-	+	-
A. suis	ATCC15557	-	+++	-	+	-
Acholeplasma lalawii	ATCC32063	-	-	-	+	-
Actinomyces israelii	ATCC10049	nd	nd	nd	nd	-
Actinomyces odontolyticulus	ATCC17929	nd	nd	nd	nd	-
Agrobacterium tumefaciens	ATCC15955	nd	nd	nd	nd	-
Arthrobacter globifiormis	ATCC08010	nd	nd	nd	nd	-
Bacillus subtilis	ATCC23059	nd	nd	nd	nd	-
Bacteroides fragilis	ATCC25285	nd	nd	nd	nd	-
Bifidobacterium dentium	ATCC27534	nd	nd	nd	nd	-
Bordetella pertussis	ATCC08467	nd	nd	nd	nd	-
Chlorobium limicola		nd	nd	nd	nd	-
Chloroflexus aurantiacus	Y400	nd	nd	nd	nd	-
Citrobacter freundii	ATCC08090	nd	nd	nd	nd	-
Campylobacter jejuni	ATCC33560	nd	nd	nd	nd	-
Clostridium clostridioforme	ATCC25537	nd	nd	nd	nd	-
Corynebacterium genitalium	ATCC33031	nd	nd	nd	nd	-
Corynebacterium glutamicum	ATCC13032	nd	nd	nd	nd	-
Corynebacterium pseudodiphtheriticum	DSM2097	nd	nd	nd	nd	-
Corynebacterium pseudotuberculosis	ATCC19410	nd	nd	nd	nd	-
Corynebacterium pyogenes	ATCC19411	nd	nd	nd	nd	-
Corynebacterium xerosis	ATCC00373	nd	nd	nd	nd	-
Corynebacterium diphtheriae	ATCC11913	nd	nd	nd	nd	-
Corynebacterium diphtheriae	ATCC13812	nd	nd	nd	nd	-

TABLE 2 (cont'd): DOT-BLOT HYBRIDIZATION OF 16S AND 23S rRNA-TARGETED PROBES

Genus, species	Target: 16S Probe: strain	1546	1547	1839	1840	23S 1984
Desulfovibrio desulfuricans	ATCC07757	-	-	-	-	-
Deinococcus radiodurans	ATCC35073	nd	nd	nd	nd	-
Erwinia	GT1751	nd	nd	nd	nd	-
Escherichia coli	N99	-	-	-	-	-
Escherichia coli	GT1665	-	-	-	+	-
Fusobacterium necrophorum	ATCC00238	nd	nd	nd	nd	-
Jonesia denitrificans	GT0666	nd	nd	nd	nd	-
K.rhinoscleromitis	GT1881	nd	nd	nd	nd	-
Klebsiella pneumoniae	ATCC13883	nd	nd	nd	nd	-
Micrococcus conoglomeratus	ATCC00401	nd	nd	nd	nd	-
Micrococcus luteus	ATCC00381	nd	nd	nd	nd	-
Mycobacterium species	GT0298	nd	nd	nd	nd	-
Mycoplasma pneumonia	ATCC15531	-	-	-	+	-
Mycoplasma putrefaciens	ATCC15718	-	-	-	+	-
Mycoplasma genitalium	ATCC33530	-	-	-	+	-
Mycoplasma hominis	ATCC23114	-	-	-	+	-
Nocardia asteroides	ATCC03308	nd	nd	nd	nd	-
Nocardia albus	DSM43109	nd	nd	nd	nd	-
Nocardia dassonvillei	DSM43235	nd	nd	nd	nd	-
Pimelobacter simplex	DSM08929	nd	nd	nd	nd	-
Pasteurella aerogenes	ATCC27883	-	++++	-	+	-
Pasteurella gallinarum	NCTC11188	-	++++	-	+	-
Pasteurella multocida	ATCC10322	-	++++	-	+	-
Pasteurella ureae	IngarOslen	-	++++	-	+	-
Propionobacterium acnes	ATCC06919	nd	nd	nd	nd	-
Propionobacterium thoenii	DMS20276	nd	nd	nd	nd	-
Psedomonas cepacia	ATCC13945	-	-	-	+	-
Rhodococcus aurantiacus	GT3589	nd	nd	nd	nd	-
Rhodococcus bronchialis	ATCC25592	nd	nd	nd	nd	-
Rhodococcus equi	GT0665	nd	nd	nd	nd	-
Rhodococcus erythopolis	ATCC19369	nd	nd	nd	nd	-
Rhodococcus fascians	ATCC12974	nd	nd	nd	nd	-
Rhodococcus ketoglutanicum	ATCC15587	nd	nd	nd	nd	-
Rhodococcus obuensis	ATCC33610	nd	nd	nd	nd	-
Rhodococcus rhodochrous	ATCC04273	nd	nd	nd	nd	-
Rhodococcus sputi	ATCC29627	nd	nd	nd	nd	-
Salmonella typhimurium	e23566	nd	nd	nd	nd	-
Spirochaeta aurantia		nd	nd	nd	nd	-
Staphylococcus aureus	ATCC12600	nd	nd	nd	nd	-
Staphylococcus aureus	GT1711	-	-	-	+	-
Streptomyces griseus	ATCC10137	nd	nd	nd	nd	-
Streptococcus faecalis	ATCC19433	nd	nd	nd	nd	-
Streptomyces mutans	GT0409	nd	nd	nd	nd	-
Streptomyces mitis	GT3490	nd	nd	nd	nd	-
Candida albicans	1008-88	-	-	-	+	-
Crytococcus neoformans		nd	nd	nd	nd	-
Human Blood		-	-	-	+	-
Human T-cells		nd	nd	nd	nd	-

TABLE 2 (cont'd): DOT-BLOT HYBRIDIZATION OF 16S AND 23S rRNA-TARGETED PROBES

Genus, species	Target: 16S Probe: 1546	1547	1839	1840	23S 1984
Mouse L-Cell	-	-	-	+	-
Normal stool	nd	nd	nd	nd	-
Wheat Germ	nd	nd	nd	nd	-

100ng RNA spotted per dot
Results were recorded after overnight exposure of autoradiographs with 2 intensifying screens
++++ = control level of hybridization; + = barely visible; and - = zero.
A. = Actinobacillus
nd = not done

These data demonstrate that for the probes derived from the 16S rRNA sequence, Probe 1546 hybridizes specifically to *Haemophilus influenza* types B, C, D and E and not to any of the other strains tested. Probe 1547 hybridizes to all *Haemophilus influenza* bacteria as well as to some other species of *Haemophilus* (*Haemophilus aphrophilus, Haemophilus avium*, a few *Haemophilus* parainfluenza isolates, and *Haemophilus segnis*). It also hybridizes to bacteria other than genus *Haemophilus* (*A. actinomycetemcomitans, A. seminis, A. equli, A. suis*, and *Pasteurella* species). Probe 1839 hybridizes to *Haemophilus influenza* types A, C, E, F, and *A. actinomycetemcomitans*.

In addition, these data demonstrate that for the probes derived from the 23S rRNA sequence, probe 1984 is highly specific and exclusive for serotypes A, B, C, D, E, and F and *Haemophilus aegyptius*. In contrast, Probe 23H1 was designed as a probe for the general detection of 23S rRNA in a sample.

Example 2: Dual Probe Liquid-Hybridization Assay

The probes of the present invention or derivatives thereof are of significant value in a variety of hybridization formats. One such format, a dual probe, sandwich-type

hybridization assay format (e.g., the homopolymer capture, dual probe, liquid hybridization format described in U.S. Patent No. 5,147,778, which is incorporated herein by reference), is used in this example. In general, in such an application, an oligonucleotide probe is modified at its 3′ terminus to contain a tract of deoxyadenosine (dA) residues ca. 20-200 residues long. This would be used to "capture" the target rRNA (following liquid hybridization) from the test sample onto a solid support (e.g., beads, plastic surface, filter, etc.) which had been suitably derivatized with poly-deoxythymidine (dT) for this purpose. A second probe is used as a detection probe and would be derivatized with a label (e.g. ^{32}P, fluorescein, biotin, etc.). In principle, the detection probe could be an oligonucleotide or a longer DNA or RNA probe. Preferably, each probe binds to contiguous areas of the target. Detection of the presence of the target nucleic acid in a test sample then is indicated by the capture of the detection probe onto the solid surface.

This could occur only if the target nucleic acid is present in the test sample. Examples of preferred capture probes are 1546 (SEQ ID NO: 1) and 1984 (SEQ ID NO: 5). Preferred detector probes are 1547 and 23HI. Preferably, probe 1546 (SEQ ID NO: 1) is used with probe 1547 (SEQ ID NO: 2) and probe 1984 (SEQ ID NO: 5) is used with probe 23HI (SEQ ID NO:6).

Other Embodiments

While the description of the invention has been made with reference to detecting rRNA of *Haemophilus influenzae*, it will be readily understood that the probes described herein and probes complementary to those described herein will also be useful for,

1) PCR amplification of ribosomal genes;

2) the detection of the genes (DNA) which specify the rRNA and, accordingly, such probes are to be deemed equivalents to the described probes and encompassed within the spirit and scope to the present invention and the appended claims; and,

3) incorporation into MDV-1 like rRNA which can be amplified by the enzyme Q-Beta replicase.

Thus, while preferred embodiments have been illustrated and described, it is understood that the present invention is capable of variation and modification and, therefore, should not be limited to the precise details set forth, but should include such changes and alterations that fall within the purview of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Shah, Jyotsna

(ii) TITLE OF INVENTION: Nucleic Acid Probes for the Detection of Haemophilus Influenzae

(iii) NUMBER OF SEQUENCES: 27

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Amoco Corporation
(B) STREET: 55 Shuman Boulevard, Suite 600
(C) CITY: Naperville
(D) STATE: Illinois
(E) COUNTRY: U.S.A.
(F) ZIP: 60563-8487

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM: MS-DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Galloway, Norval B.
(B) REGISTRATION NUMBER: 33,595
(C) REFERENCE/DOCKET NUMBER: 01886/053001

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (708) 717-2447
(B) TELEFAX: (708) 717-2430
(C) TELEX:

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCGCACTTTC ATCTTCCGAT AATACGCGGT ATT 33

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GCAGGCTTGG TAGGCATTTA CCCCACCAAC 30

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCGCACTTTC GTCTCTCGAC ACTACGCGGT ATT 33

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	33
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCACCCTTTC GTCTCTCGAC ACTACGCGGT ATT 33

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	28
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTAGTACCAC AATATGGTTT TTAGATAC 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	42
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TATCACCCTC TTTGATTGTG CTTCCCAACA CATTCTCCTA AC 42

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 53
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ACGGUAGCUA AUACCGCAUA ACGUCGCAAG ACCAAAGAGG GGGACCUUCG GGC 53

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 53
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ACUGUCGCUA AUACCGCGUA GGGUCGGGNG ACGAAAGUGC GGGACUUUAU GGC 53

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 53
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ACUGUCGCUA AUACCGCGUA UUAUCGGAAG AUGAAAGUGC GGGACUGAGA GGC 53

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 53
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ACUGUCGCUA AUACCGCGUA GUGUCGAGAG ACGAAAGUGC GGGACUUUNA GGC 53

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 52
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ACUGUCGCUA AUACCGCGUA GUGUCGAGAG ACGAAAGGGU GGGACUUUUA NC 52

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 53
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

ACUGUCGCUA AUACCGCGUA GUGUCGAGAG ACGAAAGUGC GGGACUGAGA GGC 53

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 53
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GAUUAGCUAG UAGGUGGGGU AACGGCUCAC CUAGGCGACG AUCCCUAGCU GGU 53

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	53
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GAUUAGGUAG UUGGUGGGGU AAGGGCCUAC CAAGCCGACG AUCGCUAGCU GGU 53

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	53
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GAUUAGGUAG UUGGUGGGGU AAAGGUCUAC CAAGCCUGCG AUCUCUAGCU GGU 53

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	53
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GAUUAGGUGC GGGUAGUUGG UGGGGUAAAU GCCUACCAAG CCUGCGAUCU CUA 53

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 56
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

UCAGUGUGUG UGUUAGUGGA AGCGUCUGGA AAGGCGCGCG AUACAGGGUG ACAGCC 56

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

AACAGUAUGG UUAGGAGAAU GUGUUGGGAA GCACAAUCAA AGAGGGUGAU NAUC 54

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AGCAAUAUAG UUAGGAGAAU GUGUUGGGAA GCACAAUCAA AGAGGGUGAU AAUC 54

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AAUGGUUUUG UUAGGAGAAU GUGCUGGGAA GCUCAAUCGU AGAGGGUGAU AAUC 54

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

AAUGACAGAG ACAGAGGAAC AAGCUGGGAA GCUUGGCGAC ACAGGGUGAU AGCC 54

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

AGCGACAGAG ACAGAGGAAU GUGCUGGGAA GCACAGCGAG ACAGGGUGAU AGCC 54

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CCCGUACACA AAAAUGCACA UGCUGUGAGC UCGAUGAG 38

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	38
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CCCGUAUCUA AAAACCAUAU UGUGGUACUA AGCUAACG 38

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	38
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CCCGUAUCUA AAAACCAUAU UGUGGUACUA AGCUAACG 38

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	38
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CCCGUAUCCG AAAACAUUAU UAUGGUACUA AGCUAACG 38

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	38
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CCCGUACUCG AAGUCUGUGU UAUGGUACUA AGCUAACG 38

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	38
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CCUGUUCUUG AAGUCUGGGU CGUGGUACUA AGCUAACG 38

What is claimed is:

1. An isolated nucleic acid consisting essentially of 10 to 70 nucleotides in a sequence which forms a stable hybridization duplex under normal hybridization conditions with a region of *Haemophilus influenzae* 16S rRNA or rDNA bounded by nucleotide positions 182 to 193 or 252 to 278, or a region of *Haemophilus influenzae* 23S rRNA or rDNA bounded by nucleotide positions 343 to 356, but does not form a stable hybridization duplex with any region of rRNA or rDNA non-Haemophilus organisms under the same hybridization conditions.

2. An isolated nucleic acid consisting essentially of 10 to 70 nucleotides in a sequence which forms a stable hybridization duplex with *Haemophilus influenzae* 16S rRNA or rDNA bounded by nucleotide positions 182 to 193, or a region of *Haemophilus influenzae* 23S rRNA or rDNA bounded by nucleotide positions 343 to 356, but does not form a stable hybridization duplex with any region of rRNA or rDNA or non-*Haemophilus influenzae* organisms under normal hybridization conditions.

3. The nucleic acid of claim 2, wherein said nucleic acid forms a stable hybridization duplex under normal hybridization conditions with probe 1546 (SEQ ID NO: 1), probe 1984 (SEQ ID NO: 5), or a nucleotide sequence complementary to one of said numbered probes.

4. The nucleic acid of claim 3 wherein said nucleic acid is probe 1546 (SEQ ID NO: 1), probe 1984 (SEQ ID NO: 5), or a nucleic acid complementary to one of said numbered probes.

5. A set of isolated nucleic acids comprising at least two nucleic acids, wherein each of said nucleic acids is 10 to 70 nucleotides in length and forms a stable hybridization duplex under normal hybridization conditions with a region of *Haemophilus influenzae* 16S rRNA or rDNA bounded by nucleotide positions 182 to 193 or 252 to 278, or a region of *Haemophilus influenzae* 23S rRNA or rDNA bounded by nucleotide positions 305 to 314 or 343 to 356.

6. The set of nucleic acids of claim 5, wherein when one of said nucleic acids also forms a stable hybridization duplex with the rRNA or rDNA of a non-*Haemophilus influenzae* organism under normal hybridization conditions, a second of said nucleic acids does not form a stable hybridization duplex with the rRNA or rDNA of said non-*Haemophilus influenzae* organism under the same hybridization conditions.

7. The set of claim 5 wherein said set comprises at least one of the nucleic acids of claim 1.

8. The set of claim 5, wherein said set comprises at least one of the nucleic acids of claim 2.

9. The set of claim 5, wherein said set comprises at least one of the nucleic acids of claim 3.

10. The set of claim 5, wherein said set comprises probes 1546 (SEQ ID NO: 1) and 1547 (SEQ ID NO: 2).

11. The set of claim 5, wherein said set comprises probes 1984 (SEQ ID NO: 5) and 23HI (SEQ ID NO: 6).

12. A method of detecting the presence of *Haemophilus influenzae* in a sample comprising:

a) contacting the sample with at least one nucleic acid of claim 1 under normal hybridization conditions which allow the formation of a hybridization duplex between said nucleic acid and rRNA or rDNA of *Haemophilus influenzae*, if present, and do not allow said nucleic acid to form a stable nucleic acid duplex with non-*Haemophilus* organisms; and

b) monitoring the sample contacted with said nucleic acid to detect the presence of said duplex, the presence of said duplex indicating the presence of *Haemophilus influenzae* in the sample.

13. The method of claim 12 wherein said nucleic acid forms a stable hybridization duplex under normal hybridization conditions with probe 1546 (SEQ ID NO: 1), probe 1984 (SEQ ID NO: 5), or a nucleic acid complementary to one of said numbered probes.

14. The method of claim 12 wherein said nucleic acid is probe 1546 (SEQ ID NO: 1), probe 1984 (SEQ ID NO: 5), or a nucleic acid complementary to one of said numbered probes.

15. A method of detecting the presence of *Haemophilus influenzae* in a sample comprising:

a) contacting the sample with a set of nucleic acids of claim 5 under normal hybridization conditions which allow the formation of a hybridization duplex between each of said nucleic acids and rRNA or rDNA of *Haemophilus influenzae*, if present; and

b) monitoring the sample contacted with said set of nucleic acids for the presence of said duplex, the presence of said duplex indicating the presence of *Haemophilus influenzae* in the sample.

16. The method of claim 15, wherein said set comprises probes 1546 (SEQ ID NO: 1) and 1547 (SEQ ID NO: 2).

17. The method of claim 15, wherein said set comprises probes 1984 (SEQ ID NO: 5) and 23HI (SEQ ID NO: 6).

18. A kit for detecting the presence of *Haemophilus influenzae* comprising the nucleic acid of claim 1.

19. A kit for detecting the presence of *Haemophilus influenzae* comprising the nucleic acid set of claim 5.

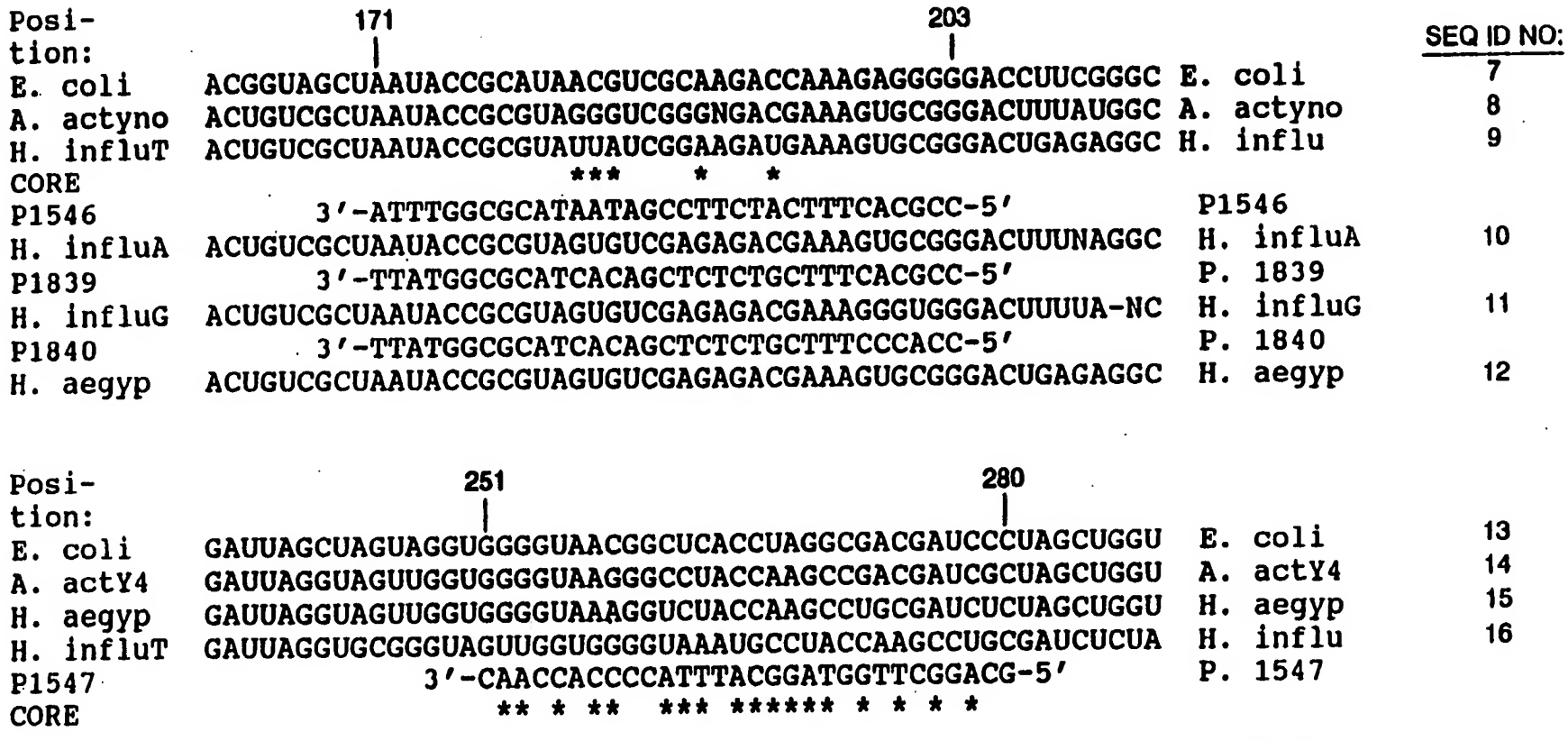


FIG. 1

23S rRNA-TARGETED PROBES AND TARGET SEQUENCES

			SEQ ID NO:
Position:	291 ... 332		
E. coli	UCAGUGUGUGUUAGUGGAAGCGUCUGGAAAGGCGCGCGAUACAGGGUGACAGCC	E. coli	17
CORE	* *		
P23HI	3′-CAATCCTCTTACACAACCCTTCGTGTTAGTTTCTCCCACTAT-5′	P23HI	
H. influF	AACAGUAU-G-GUUAGGAGAAUGUGUUGGGAAGCACAAUCAAAGAGGGUGAUNAUC	H. influF	18
H. aegyp	AGCAAUAU-A-GUUAGGAGAAUGUGUUGGGAAGCACAAUCAAAGAGGGUGAUAAUC	H. aegyp	19
H. pleur	AAUGGUUU-U-GUUAGGAGAAUGUGUUGGGAAGCACAAUCAAAGAGGGUGAUAAUC	H. pleur	20
H. parain	AAUGACAG-A-GACAGAGGAACAAGCUGGGAAGCUUGGCGACACAGGGATAUAGCC	H. parain	21
A. actyno	AGCGACAG-A-GACAGAGGAAUGUGCUGGGAAGCACAGCGAGACAGGGUGAUAGCC	A. actyno	22

Position:	338 ... 365		
E. coli	CCCGUACACAAAAAUGCACAUGCUGUGAGCUCGAUGAG	E. coli	
CORE	** ** ** * *		
P1984	3′-CATAGATTTTTGGTATAACACCATGATT-5′	P1984	
H. influF	CCCGUAUCUAAAAACCAUAUUGUGGUACUAAGCUAACG	H. influF	24
H. aegyp	CCCGUAUCUAAAAACCAUAUUGUGGUACUAAGCUAACG	H. aegyp	25
H. pleur	CCCGUAUCCGAAAACAUUAUUAUGGUACUAAGCUAACG	H. pleur	26
H. parain	CCCGUACUCGAAGUCUGUGUUAUGGUACUAAGCUAACG	H. parain	27
A. actyno	CCUGUUCUUGAAGUCUGGGUCGUGGUACUAAGCUAACG	A. actyno	28

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00802

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C07H 21/04
US CL :435/6; 536/24.32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/24.32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Journal of Clinical Microbiology, Volume 29, No. 1, issued January 1991, DALY et al, "Use of Rapid, Nonradioactive DNA Probes in Culture Confirmation Tests to Detect *Streptococcus agalactiae*, *Haemophilus influenzae*, and *Enterococcus* spp. from Pediatric Patients with Significant Infections", pages 80-82, especially page 81.	1-4, 12-14, 18 ---------- 5-10, 15, 16, 19
X --- Y	Gen-Probe, issued 1992, "AccuProbe Haemophilus Influenzae Culture Identification Test", pages 1-4, see entire document.	1-4, 12-14, 18 ---------- 5-10, 15, 16, 19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
13 APRIL 1995	24 APR 1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Carla Myers
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00802

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Journal of Clinical Microbiology, Volume 29, No. 10, issued October 1991, DAVIS et al, "Direct Identification of Bacterial Isolates in Blood Cultures by Using a DNA Probe", pages 2193-2196, especially page 2193.	1-4, 12-14, 18 ---------- 5-10, 15, 16, 19
Y	WO, A, 93/03186 (GREISEN ET AL) 18 February 1993, especially pages 19, 23 and figure 2a.	1-10, 12-16, 18, 19
Y	Journal of Bacteriology, Volume 174, issued March 1992, DEWHIRST et al, "Phylogeny of 54 Representative Strains of Species in the Family *Pasteurellaceae* as determined by Comparison of 16S rRNA Sequences", pages 2002-2013, especially figure 1.	1-10, 12-16, 18, 19
Y	EP, A, 0,272,009 (HOGAN) 22 June 1988, pages 9, 10, 23, 29, 30, 34 and 76.	1-10, 12-16, 18, 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00802

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

GenBank, EMBL and N-GeneSeq; DIALOG: Medline, Biosis, CA, World Patents
Key words: Haemophilus influenza, rRNA or ribosomal RNA, probe or primer or hybridization